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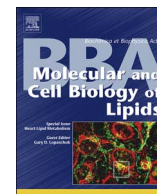
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# Phospholipid composition of packed red blood cells and that of extracellular vesicles show a high resemblance and stability during storage

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## ABSTRACT

Red blood cells (RBCs) are stored up to 35–42 days at 2–6 °C in blood banks. During storage, the RBC membrane is challenged by energy depletion, decreasing pH, altered cation homeostasis, and oxidative stress, leading to several biochemical and morphological changes in RBCs and to shedding of extracellular vesicles (EVs) into the storage medium. These changes are collectively known as RBC storage lesions. EVs accumulate in stored RBC concentrates and are, thus, transfused into patients. The potency of EVs as bioactive effectors is largely acknowledged, and EVs in RBC concentrates are suspected to mediate some adverse effects of transfusion. Several studies have shown accumulation of lipid raft-associated proteins in RBC EVs during storage, whereas a comprehensive phospholipidomic study on RBCs and corresponding EVs during the clinical storage period is lacking. Our mass spectrometric and chromatographic study shows that RBCs maintain their major phospholipid (PL) content well during storage despite abundant vesiculation. The phospholipidomes were largely similar between RBCs and EVs. No accumulation of raft lipids in EVs was seen, suggesting that the primary mechanism of RBC vesiculation during storage might not be raft-based. Nonetheless, a slight tendency of EV PLs for shorter acyl chains was observed.

## 1. Introduction

During storage, red blood cells (RBCs) undergo several biochemical and morphological changes affecting their quality and function, leading to altered membrane lipid bilayer asymmetry and to accumulation of extracellular vesicles (EVs) and cell waste in the storage medium. These changes are referred to as storage lesion [1–3]. The erythrocyte membrane is a lipid bilayer consisting of asymmetrically distributed phospholipids (PLs), sphingolipids, cholesterol, and proteins. RBCs lack a nucleus and are incapable of de novo lipid and protein synthesis and therefore depend on lipid exchange and acyl chain remodeling as the mechanisms of PL repair and renewal [4,5]. The essential membrane PL asymmetry is actively maintained by various lipid-translocating enzymes. Nevertheless, metabolic processes of RBC membranes are heavily affected by cold blood bank storage.

RBCs have a special biconcave disc shape for maximization of the

surface area and flexibility. Membrane damage to RBCs disturbs rheological properties and increases fragility after transfusion possibly leading to increased hemolysis and fast removal of the transfused nonviable RBCs from the circulation [6]. Energy depletion and oxidative damage to membrane proteins during RBC storage cause aberrations in the membrane microstructure. The correct membrane bilayer lipid asymmetry is lost, which has been postulated as an essential mechanism of RBC vesiculation [7]. EVs, like their parent cells, possess a membrane bilayer. EVs may act as bioactive effectors: they transport molecules between cells, carry membrane antigens, and take part in signaling and affect several biological processes in recipient cells [8,9]. Accumulating EVs are suspected to have inflammatory, immunomodulatory, and procoagulant effects on blood recipients [10–15], potentially weakening the overall benefits of transfusion.

Phosphatidylserine (PS) is normally found only on inner leaflets of cell membranes and when exposed, functions as a signal for

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phagocytosis and activation of coagulation [16]. Thus, removal of the externalized PS from RBCs via vesiculation may serve as a survival mechanism for RBCs [7], and in theory, can lead to enrichment of certain PS species in EV membranes. Besides, functionally active lipid rafts, enriched in e.g. cholesterol and sphingolipids [17] are believed to be involved in RBC vesiculation, meaning that there could be controlled inclusion of specific lipids into EVs. Lipid raft-associated proteins have been shown to accumulate in RBC EVs during storage [18–21], and sphingomyelin (SM) has been generally reported to get enriched in EVs of various origins compared to their parent cells [8,22].

The lipid composition of the erythrocyte membrane is thoroughly characterized at the PL class level as the erythrocyte has been used as a model cell in biomembrane lipidomic studies [23–25]. PL composition at the molecular species level has been less studied [26–28], and a storage time-dependent study on RBCs and corresponding EVs is lacking. Accordingly, we aimed to study the time course of changes in PL classes and species of packed RBCs and the corresponding EVs during a clinically relevant storage period. We were interested in possible lipid alterations in RBC membranes as a mechanism underlying RBC storage lesion. We hypothesized some differences exist in PL composition between EVs and RBCs in the course of RBC product aging.

## 2. Materials and methods

### 2.1. Packed RBCs

**Ethical considerations:** Only RBC concentrates that were not needed for clinical use were used in the study. The research use is in accordance with the rules of the Finnish Supervisory Authority for Welfare and Health (Valvira).

We analyzed altogether 10 standard leukoreduced RBC concentrates of blood type B+, obtained from the Finnish Red Cross Blood Service (FRCBS), stored in the saline-adenine-glucose-mannitol (SAGM) additive solution with citrate-phosphate-dextrose (CPD) anticoagulant at 2–6 °C under standard blood bank conditions. The maximum storage duration for RBC products in Finland is 35 days. First pilot storage experiment consisted of four and the second six RBC concentrates. Samples were taken at 2, 8, 15, 22, and 36 days of age of the blood products (Fig. 1). At each time point, the RBC concentrates were thoroughly mixed, and 3 mL of RBCs was aseptically drawn from the bag for routine in vitro quality control testing, and 17 mL for other analyses. For collection of RBC lipid samples, 0.5 mL of the original RBC concentrate was resuspended in 2 mL of the SAGM solution and centrifuged for 20 min at room temperature (RT) and 805 × g without a brake (Eppendorf centrifuge 5810R). Pelleted RBC samples were stored at –80 °C under argon in glass vials until lipid extraction.

Quality control measurements for RBC concentrates are described in

Supplementary methods.

### 2.2. EV isolation and characterization

#### 2.2.1. EV isolation

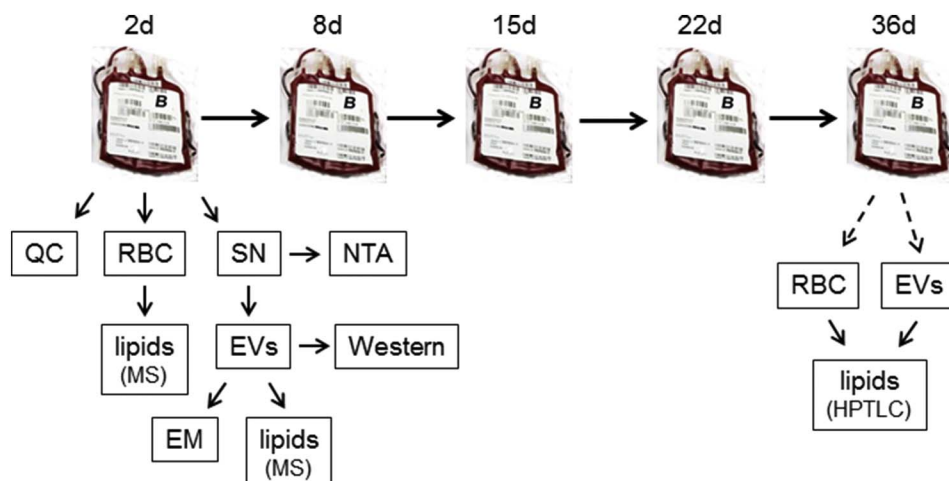
The EVs were collected and analyzed only from the second storage experiment ( $n = 6$ ). For EV collection and for removal of the cells, 15 mL of an RBC concentrate was diluted with 15 mL of phosphate-buffered saline (PBS, Sigma-Aldrich, Darmstadt, Germany) and centrifuged two times for 20 min at 805 × g and RT without a brake. The resulting supernatant was ultracentrifuged for 1 h at 100000 × g (+4 °C, fixed-angle MLA-50 rotor, Beckman Coulter, Indianapolis, IN, USA) to obtain EVs. The EVs were washed once with PBS, ultracentrifuged for 1 h at 100000 × g (+4 °C, swing out MLS-50 rotor, Beckman Coulter) and resuspended in 1 mL of PBS and stored at –80 °C in aliquots for further analysis. For electron microscopy (EM), EVs were isolated from additional RBC concentrates ( $n = 4$ ) early (2-d) and late (36-d) time point of storage and prepared freshly without freezing.

#### 2.2.2. Nanoparticle tracking analysis

To study the concentration and size distributions of EVs in RBC samples ( $n = 6$ ), 0.5 mL of the original RBC concentrate was resuspended in 2 mL of the SAGM solution and centrifuged two times for 20 min at RT and 805 × g without a brake (Eppendorf centrifuge 5810R). The resulting supernatant was analyzed for particle content by nanoparticle-tracking analysis (NTA; instrument LM14C with violet laser 405 nm, 70 mW, Malvern Instruments Ltd., Malvern, UK) and a sCMOS camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). The samples were injected manually, and data acquisition was conducted at ambient temperature in triplicate using the NanoSight software v. 2.3 (Malvern Instruments Ltd.). Settings for data acquisition were as follows: basic, camera level 14, autosettings off, polydispersity and reproducibility high, with particles per image 40–100 (acquisition time 90 s). Data were analyzed in the NanoSight software v. 3.0 (Malvern Instruments Ltd.) with threshold 5 and gain 10.

#### 2.2.3. Western blot

Pelleted EV samples (2 and 36 d) were further characterized by western blotting for the origin of EVs. Equal amounts of protein (2.5 µg) were loaded and run on a TGX Mini-Protein 10% gel (Bio-Rad Laboratories, Hercules, CA, USA) and subsequently electrophoretically transferred to nitrocellulose membranes (BioRad). Nonspecific binding was blocked with either 5% milk-0.1% Tween 20-PBS or 3% BSA-0.05% Tween 20-PBS (overnight, +4 °C). Rabbit anti-human CD235a (1:1000 dilution, clone EPR8200, Abcam, Cambridge, UK), mouse anti-human Hb (1:5000, clone 7E1F, Abcam), mouse anti-human CD61 (1:1000,



**Fig. 1.** The experimental set up. First storage experiment consisted of four and the second six red blood cell (RBC) concentrates. Extracellular vesicles (EVs) were collected and analyzed only from the second storage experiment ( $n = 6$ ). RBC concentrates ( $n = 10$ ) were stored under standard blood banking conditions. Samples were taken at 2, 8, 15, 22, and 36 days of age of the blood products. Mass spectrometric phospholipid (PL) analysis of RBCs ( $n = 10$ ), quality control (QC) measurements ( $n = 10$ ), and nanoparticle-tracking analysis (NTA,  $n = 6$ ) were conducted at each time point. EV ( $n = 6$ ) PL analysis was performed on 2-d, 15-d, and 36-d samples, and western blot on 2-d and 36-d samples. EV samples for electron microscopy (EM) were collected at 2 and 36 days of storage ( $n = 4$ ). High-performance thin-layer chromatography (HPTLC) analysis of PL classes from RBC ( $n = 9$ ) and EV ( $n = 7$ ) samples was performed at late time point (33–36 d) of storage only.

clone VI-PL2, BD), mouse anti-human apolipoprotein A1 (Apo-A1; 1:1000, Medix Biochemica, Espoo, Finland), and mouse anti-human  $\beta$ -actin (1:1000, clone AC-74, Sigma) antibodies were incubated in either 5% milk-0.1% Tween 20-PBS or 3% BSA-0.05% Tween 20-PBS (1 h, RT), followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG (H + L) secondary antibodies (1:3000 dilution, 1 h, RT, Bio-Rad Laboratories). The enhanced chemiluminescence (ECL) reagent/kit (GE Healthcare, Amersham, Little Chalfont, UK) was used for detection of results.

#### 2.2.4. Scanning electron microscopy (SEM)

EVs were collected as described in subsection 2.2.1 from four RBC concentrates after 2 and 36 d of storage and processed the same day. In total, 50–75  $\mu$ L of concentrated EVs was placed on concanavalin A (Sigma-Aldrich)-coated glass cover slips and fixed with 5% glutaraldehyde (G7651 Sigma, EM quality) in 0.05 M HEPES buffer (pH 7.2) for 30 min and washed repeatedly with 0.05 M HEPES buffer and stored in a buffer solution until the next day. Samples were post-fixed with 1% OsO<sub>4</sub> and dehydrated in an increasing gradient of ethanol. Samples were allowed to dry on hexamethyldisilazane and were imaged immediately. Before microscopy, the samples were coated with platinum. SEM analyses were conducted by means of a FEG-SEM Quanta 250 microscope (FEI Company, Hillsboro, OR, USA). Diameter of EVs was measured manually in Fiji platform (ImageJ 1.51n, <http://imagej.nih.gov/ij>) from 2-d ( $n = 156$  EVs) and 36-d ( $n = 154$  EVs) images ( $n = 3$  individuals in both time points).

#### 2.3. Sample preparation for mass spectrometry

Lipids were extracted from RBCs, first from four and subsequently from six RBC concentrates (altogether  $n = 10$ ) stored for 2, 8, 15, 22, and 36 days. From the second storage experiment corresponding EVs ( $n = 6$ ) were collected (2, 15, and 36 days) by the Folch extraction of total lipids [29]. Briefly, the RBC pellet, approximately 0.5 mL, was mixed with 3 mL of MeOH. Next, 0.45 mL of H<sub>2</sub>O and 3 mL of MeOH was added to the sample and mixed thoroughly. One mL of the sample was mixed with 0.32 mL of H<sub>2</sub>O and 2.2 mL of MeOH, followed by lipid extraction as described elsewhere [29]. The EV sample, 0.25 mL in PBS, was diluted with water to a final volume of 0.45 mL before the lipid extraction.

#### 2.4. Electrospray ionization mass spectrometry of PL species

The analytical methods used for PL species profiling followed in principle the guidelines published previously [30,31]. A 6490 Triple Quad LC-MS/MS system (Agilent Technologies, Santa Clara, CA, USA) was used, and the lipid extracts of the RBCs and EVs were infused into the mass spectrometer (MS) in chloroform/methanol (1:2) with 1% NH<sub>4</sub>OH; the lipid species were detected by means of the class-specific MS/MS scans [32]. The sample solutions were introduced into the MS ion source by a Harvard syringe pump at a steady rate of 10  $\mu$ L/min, and nitrogen served as the nebulizing and drying gas. The EV samples contained lipophilic compounds suppressing lipid ionization during direct infusion into the ion source of the MS apparatus. Running the EV samples with LC pre-separation [a C18 column and common ammoniated acetonitrile-isopropanol solvent system, details in [33]] diminished the artefact effects and allowed for more sensitive detection by means of the class-specific MS/MS scans. To verify the comparability of different methods, RBC samples ( $n = 6$ ) containing the same internal standards were analyzed by both the direct infusion and the LC-MS/MS method. The samples were spiked with the same appropriate cocktail of internal standards enabling correction for the effects of carbon chain lengths on the detector response. The potentials of the spray needle, capillary, and electric guides, and the collision energies were optimized separately for different lipid classes. Full-scan mass spectra ranging from  $m/z$  400 to 1000 were collected in both positive and negative

ionization modes. In addition to the MS<sup>+</sup> and MS<sup>−</sup> scans, an MS/MS precursor ion scan was used to detect the diacyl species of phosphatidylcholine (PC) and SM (precursors for the fragment ion  $m/z$  184), and MS/MS neutral loss (NL) scans were applied to detect the diacyl species of phosphatidylethanolamine (PE) (NL141) and PS species (NL87). The saved spectra were averaged using the Mass Hunter software (Agilent). Further data analysis for RBCs was performed in the LIMSA software [34].

#### 2.5. High-performance thin-layer chromatography (HPTLC) of lipid classes

Relative composition of the major lipid classes in RBCs and EVs was determined by HPTLC. Samples were collected from additional RBC concentrates at 33–36 days of age ( $n = 9$ ). EVs were isolated from 100 mL and RBCs from 0.2 mL of a blood product, and lipids were extracted as described above. Silica plates (HPTLC Silica Gel 60 F<sub>254</sub>, 20 × 10 cm, Merck, Darmstadt, Germany) were prewashed with a blank development solution (chloroform/methanol 1:1) and were dried for at least 48 h in a desiccator. Sample aliquots (20  $\mu$ L, in chloroform/methanol 1:2) and standards for each lipid class were applied onto silica plates by a CAMAG Automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland). The PLs were separated on the plates in a horizontal developing chamber by means of chloroform/methanol/acetic acid/water (60:50:1:3.5) as the eluent, and the free cholesterol was separated on another plate by hexane/diethylether/acetic acid/water (26:6:0.4:0.1). The plates were stained by dipping them into a 3% CuSO<sub>4</sub>·5H<sub>2</sub>O + 8% H<sub>3</sub>PO<sub>4</sub> aqueous solution and by heating them on a hot plate until visible sample bands appeared. The lipids were quantified by scanning the plates on a ChemiDoc Touch Imaging System (Bio-Rad) and comparing the signal areas to those of authentic lipid standards (Avanti Polar lipids, Alabaster, Alabama, USA) for each lipid class.

#### 2.6. Statistical analyses

To study the statistical significance of PL class changes and molecular species changes during storage, the related-samples Friedman test for one-way repeated-measures analysis of variance was used, and to compare independent samples, the Mann-Whitney  $U$  test was used. For NTA and SEM measurements the Mann-Whitney  $U$  test was used. Differences with a  $P$  value < 0.05 were considered statistically significant. Analysis was performed in SPSS v.23.0 (IBM Corp., Armonk, NY, USA).

### 3. Results

#### 3.1. Quality control of packed RBCs

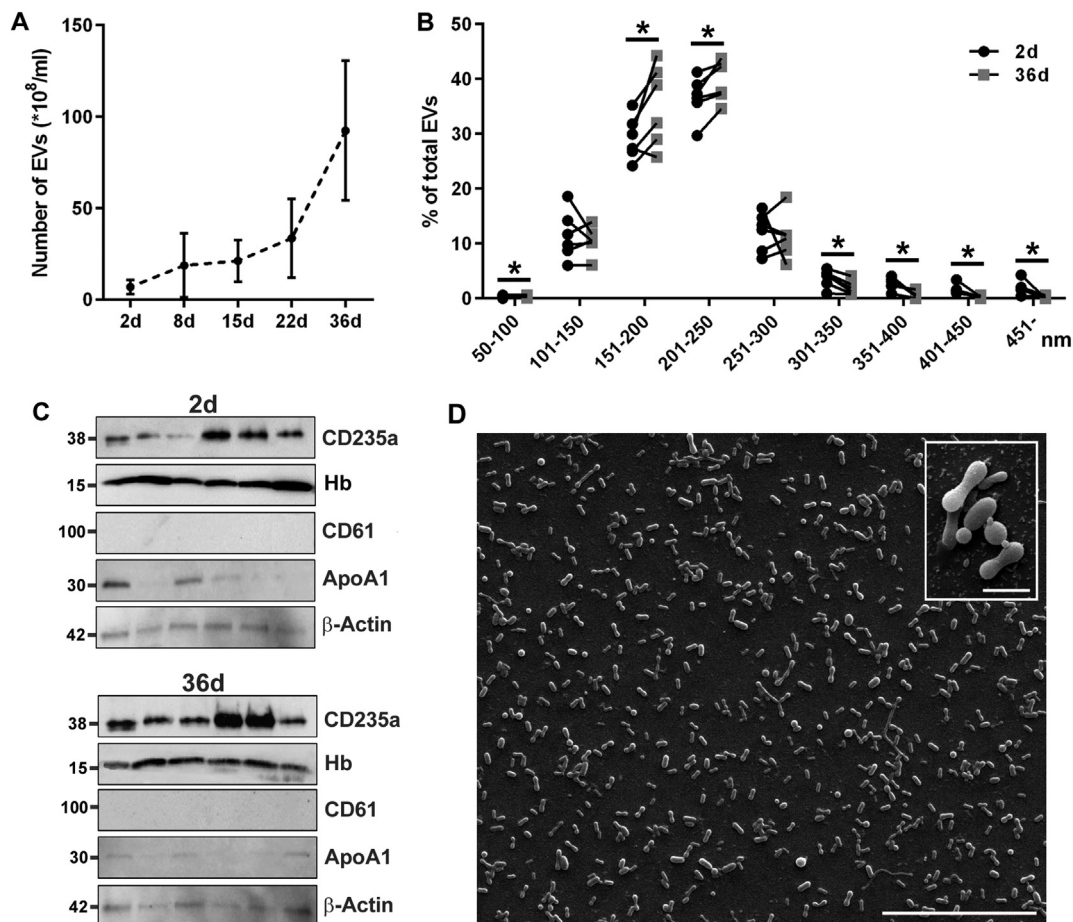
Comparable changes were observed in quality control measurements during storage relative to those described in the literature (described in more detail in Supplementary results, Supplementary Fig. 1).

#### 3.2. Characterization of EVs

The numbers of particles varied substantially among individual blood concentrates. In all six samples, we found that the particle concentration increased during storage (Fig. 2a). Median particle concentration was  $5.39 \times 10^8$ /mL (day 2),  $17.48 \times 10^8$ /mL (day 15), and  $86.29 \times 10^8$ /mL (day 36). The greatest increase in the particle concentration was between days 22 and 36 of storage. In the size distribution of particles, we observed a statistically significant shift toward a smaller vesicle size during storage (Fig. 2b). Most of the particles (90%) were 100–300 nm in size. In fresh (compared to 36-d) samples, there were significantly more particles sized over 300 nm, but also slightly more small particles sized < 100 nm.

To evaluate the origin of EVs in the RBC concentrates, we studied





**Fig. 2.** EV characterization. EV concentration and size distribution were evaluated in an RBC-free supernatant by nanoparticle-tracking analysis (NTA) ( $n = 6$ ). EV concentration increased considerably during storage (A) and a statistically significant shift toward a smaller EV size was observed (size in nm) (B). Western blot analysis of day 2 and day 36 EV samples shows that CD235a and hemoglobin (Hb) were present in EVs, while CD61 was absent, and ApoA1 amounts were negligible. β-actin was used as a loading control. (C). Scanning electron microscopy reveals a wide variety of shapes and sizes in EVs from a 36-day-old RBC concentrate, scale bar 1 μm. Inset is from 2-day-old RBC concentrate, scale bar 0.5 μm. (D). NTA results are shown as mean  $\pm$  SD, and statistical significance was analyzed by the Mann-Whitney  $U$  test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

the expression of the RBC surface marker CD235a, Hb, the platelet surface marker CD61, and the high-density lipoprotein (HDL) marker apolipoprotein A1 (ApoA1) in the EVs by western blotting. The RBC marker CD235a and Hb were present in all the samples (Fig. 2c). Platelet marker CD61 was not visible in the RBC EVs, while in the platelet EV sample, it was clearly present (Supplementary Fig. 2). In addition, a minor ApoA1 band was detected, especially in some 2-d samples (Fig. 2c). Nevertheless, the amounts of the other markers did not seem increase during storage; therefore, the EVs in the older RBC concentrates were mostly of RBC origin. Some minor contamination with EVs from other cells can be present due to a small residue of plasma in the production process (maximum 20 mL per RBC concentrate).

SEM was performed on EVs from fresh (2 d) and aged (36 d) RBC concentrates ( $n = 4$ ). The amount of EVs was negligible in samples from early storage, but different sizes and shapes were still evident (Fig. 2d inset). The samples from late storage contained a lot of EVs of a wide variety of shapes and sizes (Fig. 2d). Mean diameter and also the diversity of 2-d EVs (352.6 nm,  $\pm$  215.6) was greater than that of 36-d EVs (266.0 nm,  $\pm$  93.2) (Supplementary Fig. 3).

### 3.3. PL composition of RBCs and EVs

The PL profiles of RBCs remained rather constant during storage (2–36 d) and no statistically significant differences in the lipid profiles were seen at the class level as analyzed by ESI-MS/MS (Supplementary Fig. 4,  $n = 10$ ). These results are comparable to the reported results for

RBCs from fresh blood [26]. The EV samples contained substances that interfered with the MS detection of phosphatidylethanolamine plasmalogen species (PEp), for which no sensitive specific MS/MS detection mode exists. Therefore, the relative composition of the four major PL classes (PC, PE, PS, and SM) of EVs and RBCs was then compared by HPTLC at a late time point of storage (33–36 d). These samples contained only low concentrations of lipoprotein and platelet markers and thus were the purest RBC-derived EVs. There were no significant differences between the PL class profiles of EVs and RBCs, and no enrichment of PS or SM in EVs was found (Fig. 3). In addition, cholesterol/PL ratio was not statistically different between RBCs and EVs ( $0.64 \pm 0.02$  compared to  $0.52 \pm 0.12$ , respectively,  $n = 4$ ). In general, EV lipid composition had more variation between individual donors.

During storage, the molecular species profiles of RBCs showed few statistically significant changes in the main PL classes (Fig. 4), but the absolute shifts were no more than 2 mol% in any one of the PL species. The changes in the individual PL species owing to storage were more numerous in EVs than in RBCs (Fig. 4). In general, the molecular species of PC and SM, enriched on the outer leaflet of the membrane were altered more than the molecular species of PE and PS, found predominantly on the inner leaflet of the membrane [25]. A subtle tendency for PLs with relatively short acyl chains at the expense of PLs with longer unsaturated chains was observed in EVs during the storage in comparison with RBCs. The differences, though statistically significant in some details, remained minor in general. There was high

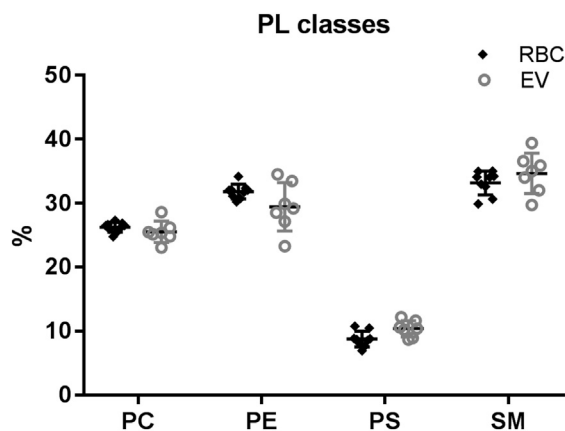


Fig. 3. The relative amounts of four major phospholipid (PL) classes of the RBCs ( $n = 9$ ) and the EVs ( $n = 7$ ) collected from RBC concentrates at 33–36 d of storage. Lipids were analyzed by high-performance thin-layer chromatography. The values are weight percentages for each class when the sum of the four main PL classes was set to 100%. PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine.

individual variation among EV samples, especially at the early time points, possibly reflecting the presence of varying amounts of lipoprotein and platelet material (Fig. 2c).

Both RBCs and EVs had largely similar PL molecular species composition. We focused on the comparison of 36-d EVs and RBCs owing to the best purity of the EV samples at the end of the storage, when they were mainly composed of RBC-derived EVs (Fig. 5). The largest differences between EVs and RBCs and the largest individual variation were seen in PE diacyl species. The most remarkable difference on day 36 was in PE 38:4, whose amount was found to be substantially less in EVs than in RBCs. Additionally, less PE 38:7, PE 36:5, and PE 36:2 and

respectively more PE 36:1 and PE 36:4 was detected in EVs compared to RBCs. The PS species profiles were the most stable during storage, and only negligible differences between EVs and RBCs were observed. In SM species, there was significantly more SM 16:1 and SM 24:0 in EVs and more SM 22:1 and SM 24:2 in RBCs. A slight general pattern of favoring shorter chains in EV PLs and longer chains with a higher degree of unsaturation in RBC PLs were seen in each PL class.

#### 4. Discussion

The PL compositions of our blood bank-stored RBCs well matched the known RBC lipidomes described at the class level [24,25] and at the species level for RBCs obtained from fresh blood [26]. Contrary to our expectations, we did not detect any major differences in PL class and species compositions between stored RBCs and the corresponding EVs. Thus, no systematic PL content alterations could be detected in RBCs as a sign of storage lesion.

RBCs exchange PLs, outer-leaflet lipids PC and SM in particular, with plasma lipoproteins, and a diet change or aberrations in plasma lipoproteins induce changes in RBC membranes. The lipid turnover rate in RBCs is slow, and it takes up to one month to see diet-induced changes in vivo [4,5]. The acyl chains of PS and PE, residing in the inner membrane leaflet, can be slowly replaced via ATP-dependent incorporation mechanisms [35]. In cold storage, ATP depletion is known to take place [1], and lipid metabolism is probably slowed down. During storage, we observed substantially more alterations in the outer-leaflet PL profiles, while composition of the inner-leaflet PL species remained more stable both in EVs and RBCs. This finding is in agreement with the fact that in stored RBC concentrates, there is no de novo source of PLs, but the material for lipid exchange and fatty-acid acylation is limited to existing membrane and lipoprotein particles present in the blood product during RBC collection.

During early RBC storage, the amount of EVs in general is miniscule,

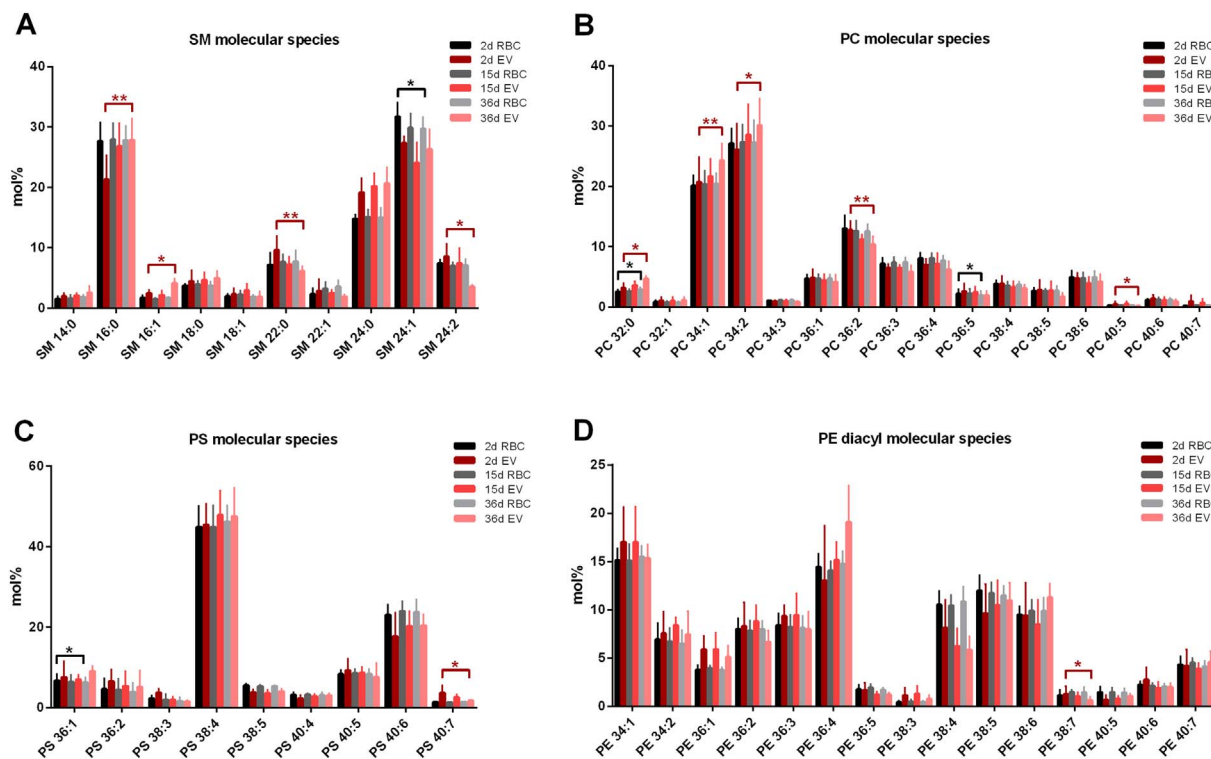


Fig. 4. Molecular species of four major phospholipid (PL) classes in RBCs ( $n = 10$ ) and EVs ( $n = 6$ ) at 2-d, 15-d, and 36-d time points of storage. More numerous statistically significant changes as a function of storage duration are observed in outer-leaflet sphingomyelin (SM) (A) and phosphatidylcholine (PC) diacyl species (B) than in phosphatidylserine (PS) (C) and phosphatidylethanolamine (PE) diacyl species. Results are shown as mean molar percentages (mol %  $\pm$  SD). Statistical significance was analyzed by Friedman's test for one-way repeated-measures analysis of variance (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , red color indicates EVs, and black color RBCs).

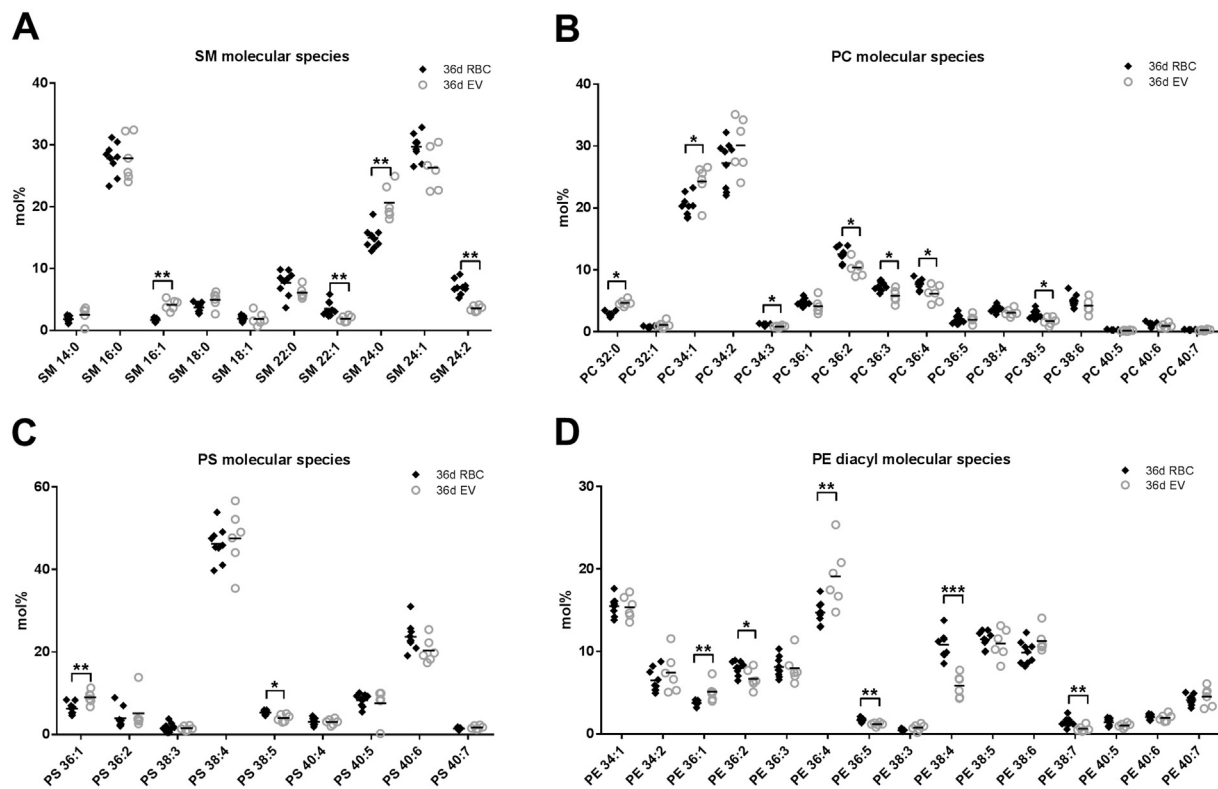


Fig. 5. A comparison of molecular species of four major phospholipid (PL) classes in RBCs ( $n = 10$ ) and EVs ( $n = 6$ ) at 36 d of storage. Results are shown as mean mol%  $\pm$  SD. Statistical significance was assessed by the Mann-Whitney  $U$  test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

and possible contaminating lipoproteins and EVs from cell sources other than RBCs make a relatively larger contribution to the lipid content. Some lipoprotein particles from residual plasma are likely to remain in the EV aliquot during the ultracentrifugation process [36]. As proof, we did find a faint band of the HDL marker Apo-A1 in our EV samples, manifesting more at an early time point. It has been suggested that stored RBC concentrates may contain a substantial proportion of platelet-derived EVs [14], especially in the beginning of storage, because platelets are known to vesiculate actively. Nevertheless, we believe that this is unlikely, especially later in storage, because the platelet contamination compared to RBC mass is minimal. In addition, we did not detect platelet marker CD61 in our EV samples. The similarity of PLs between RBCs and the associated EVs adds to the proof that the majority of EVs found in RBC products are RBC-derived. In the later storage, the relative amount of RBC-derived EVs became much greater and the significance of possible contaminating lipids decreased; therefore, we consider the lipid data from 36-d EVs more reliable.

We did not observe significant differences between the PL class compositions of EVs and RBCs. Accumulation of PS in EVs was not observed, indicating no augmented removal of PS from RBCs via vesiculation. In fact, the changes in PS species with respect to time (or when comparing RBC and EV PS profiles) were the smallest. Despite the reported accumulation of lipid raft-associated proteins in stored RBC EVs [18–21], our data indicate no enrichment of raft-associated lipid SM in EVs, as has been suggested for exosomes, which are EVs of endosomal origin [22]. Furthermore, we did not observe enrichment of cholesterol, another raft-associated lipid, in EVs compared to RBCs. Thus, based on our results, raft-based process does not seem to be the major mechanism in storage-associated RBC vesiculation. Unfortunately, we were not able to study molecular distribution of PLs or changes in lipid asymmetry, as that has been proposed as an essential mechanism of RBC vesiculation during storage [7]. Nevertheless, that may not necessarily cause major, systematic differences in PL composition between EVs and RBCs, at least according to our data. This is different from the

selective sorting of proteins seen in RBC EVs during aging and, e.g., band 3- or protein oxidation-related mechanisms for vesicle generation from RBCs have been suggested [37].

PL profiles of RBC molecular species and of the corresponding EV molecular species have not been extensively compared before. Stored human RBCs and their EVs have been previously compared by only one group, who reported differences in PS molecular species profiles of RBCs and EVs in an outdated product [27]. They stated that 38:1 is the main PS species in aged packed RBCs. The fact that RBCs analyzed in their study were very old, could explain their contradictory finding to ours. Large amounts of carbon chains this long with only one double bond may lower membrane fluidity and would not be expected to be favorable in cell membranes. In addition, 20- or 22-carbon saturated or monounsaturated fatty acids, which most likely comprise one of the two chains in PS 38:1, have not been found in significant amounts in RBCs [3,23,35,38,39]. Moreover, we did not detect a significant amount of this PS species in RBCs at any time point, but instead a large relative amount of PS 38:4, in line with another study [26]. The lipid profile of EVs collected from outdated RBC product reported by Bicalho et al. is substantially similar to the lipid profiles of both our RBCs and EVs [27].

In EVs, the PL acyl chains were slightly shorter than those in RBCs. This result could be explained by the differences between the surface areas of the two membrane leaflets, where the difference is much larger in the small EVs compared to the parent cells. When the relative area of the inner leaflet gets smaller, it should be seen as a relative loss of cone-shaped PLs such as PE species with long and highly unsaturated acyl chains, e.g., PE 38:4. This membrane curvature effect could theoretically lead to some physicochemically-driven selection of lipids to accomplish energetically favorable packing of the membrane [40–42]. In addition, the shorter chains, even one, influence the efflux properties of the PL molecules. For example, PE 36:4, which was favored in EVs at the expense of PE 38:4, has a higher chemical activity than 38:4 does, and is preferred as a substrate by phospholipases [43]. This phenomenon is worth noticing when addressing the biological effects of



transfused EVs.

We saw EVs of varying morphology. It is possible that EVs of different size and morphology have different functional qualities. PL shape, size, and charge affect their tendency to form bilayer membranes of different curvature, and also the associated membrane proteins influence on the tendency to form certain shapes [40]. Therefore, EVs of different morphology may consist of different types of PLs and proteins. We were not able to separate and isolate EVs with different morphological features to perform further analysis. Some of the EVs seemed to be about to split in half or budding, thus creating smaller daughter EVs, but this phenomenon could not be solidly confirmed by SEM imaging. The EV size distribution shifted toward smaller EV sizes during storage, in line with this theory, supported both by NTA and SEM results. Contrary to our results, it has been proposed that RBC EVs increase in size during storage and fusion might be one possible mechanism [20,44]. EV shape and formation may be a dynamic process, and EV shapes may constantly change in the medium. Additionally, the ultra-centrifugation process may cause alterations in EV morphology [45].

Our study has some limitations. Mass spectrometric analysis of EVs was challenging due to a very low amount of the sample. In particular, the negative ionization mode of the MS, less sensitive to start with than the positive ionization mode, was hampered by the presence of contaminants. On day two, the amount of EVs, and thus the material being analyzed, was much smaller as compared to day 36, thereby leading to different intensity of the MS signals. This effect caused higher deviation in the day 2 data for methodological reasons. Owing to the small amount of the EV material, we were able to include only three time points and analyze only the four largest PL classes and had to leave out more minor groups such as bioactive lyso-PC and ceramides, although those could have functional importance. PEp species could not be reliably analyzed in EVs because of the lack of a specific detection mode sensitive enough. In addition, we were only able to study a pool of all EVs with different sizes and morphologies, thus, the possible compositional changes in PLs in different EV subtypes during storage of RBCs concentrate requires further studies.

The strengths of our study: We analyzed the four major PLs of both RBCs and EVs derived from the same RBC concentrates from early, middle and late time points of storage within the Finnish clinical usage period to enable a rational comparison. Species level PL analysis of packed RBCs has been done only by one other research group with partly conflicting results [27,28]. In our study, several appropriate MS scans were run for each type of lipid to obtain reliable results not biased by adducts and other contaminants. Because of challenges in the MS analysis of PE plasmalogen species in EVs, the PL class level comparison between RBCs and EVs was carried out by HPTLC.

## 5. Conclusions

Our results indicate abundant RBC-derived vesiculation, and although greatly increasing during storage, vesiculation does not seem to disturb RBC membrane PL composition of the four major PL classes. The lipid content of RBCs and the corresponding EVs bear a high resemblance. During storage, in EVs, a subtle tendency for accumulating PL species with shorter chains was observed. There was no accumulation of raft lipids in EVs, indicating that the main mechanism of RBC vesiculation during storage may not be raft-based. The PL content of RBCs seems to stay relatively unchanged during clinical storage time, suggesting that PL compositional alterations may not be among the main mechanisms behind RBC storage lesion.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbalip.2017.09.012>.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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